

## ● INVITED REVIEW

# Protective effects of gonadal hormones on spinal motoneurons following spinal cord injury

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**Funding:** This work was supported by grants from Indiana Spinal Cord and Brain Injury Research Fund (ISCBIRF) and by IU's Office of the Vice Provost for Research through the Faculty Research Support Program to DRS, and NIH R01 NS103481, R01 NS100531, Department of Veterans Affairs I01 RX002356, I01 BX003705, Craig H Neilsen Foundation 296749, Indiana Department of Health 019919, ISCBIRF, and Mari Hulman George Endowment Fund to XMX.

## Abstract

Spinal cord injury (SCI) results in lesions that destroy tissue and disrupt spinal tracts, producing deficits in locomotor and autonomic function. The majority of treatment strategies after SCI have concentrated on the damaged spinal cord, for example working to reduce lesion size or spread, or encouraging regrowth of severed descending axonal projections through the lesion, hoping to re-establish synaptic connectivity with caudal targets. In our work, we have focused on a novel target for treatment after SCI, surviving spinal motoneurons and their target musculature, with the hope of developing effective treatments to preserve or restore lost function following SCI. We previously demonstrated that motoneurons, and the muscles they innervate, show pronounced atrophy after SCI. Importantly, SCI-induced atrophy of motoneuron dendrites can be attenuated by treatment with gonadal hormones, testosterone and its active metabolites, estradiol and dihydrotestosterone. Similarly, SCI-induced reductions in muscle fiber cross-sectional areas can be prevented by treatment with androgens. Together, these findings suggest that regressive changes in motoneuron and muscle morphology seen after SCI can be ameliorated by treatment with gonadal hormones, further supporting a role for steroid hormones as neurotherapeutic agents in the injured nervous system.

**Key Words:** testosterone; dihydrotestosterone; estradiol; steroids; atrophy; neuroprotection; morphology; dendrites; muscle fibers; retrograde labeling

## Introduction

Spinal cord injury (SCI) is a devastating medical problem with high mortality and long-term morbidity. The number of SCI patients in the US who were alive in 2017 is between 245,000–353,000, with an annual incidence of 17,500 new cases; the estimated lifetime cost of SCI is \$1.6–4.8M per patient (National Spinal Cord Injury Statistical Center (NSCISC), 2018).

The pathophysiology of SCI is complex, and after the initial mechanical deformation, a protracted period of progressive damage occurs, causing spreading of the lesion and further segmental destruction (Liu et al., 1997). A variety of mechanisms contribute to this progressive secondary injury, including excitotoxicity (Liu et al., 1991), free radical generation (Diaz-Ruiz et al., 2002), protease activation (Wang et al., 1997), and inflammation (Ritz and Hausmann, 2008; Liu et al., 2009; Liu and Xu, 2010), resulting in the death of motoneurons, interneurons, and glial cells in the spinal cord (Liu et al., 1997; Liu and Xu, 2010). Similarly, damage to spinal nerves resulting in laceration and avulsion of spinal roots (e.g., cauda equina injury with high impact motor vehicle accidents; Moschilla et al., 2001) can lead to the death of motoneurons and preganglionic autonomic neurons in the spinal cord, resulting in autonomic and motor dysfunction (Hoang et al., 2003).

## Surviving Motoneurons as a Treatment Target

The majority of treatment strategies after SCI have concentrated on the damaged spinal cord, for example working to reduce lesion size or spread, or encouraging regrowth of severed descending axonal projections through the lesion, hoping to re-establish synaptic connectivity with caudal targets. We have focused on a novel target for treatment after SCI, surviving spinal motoneurons and their target musculature. In contrast to the extensive studies on neuroprotection and axonal regeneration at the lesion site, the morphological and functional consequences of SCI for surviving motoneurons have been significantly understudied. The spinal motoneurons are the final common pathway for motor output to the effector muscles, and any impairment in these motoneurons can cause paralysis and muscle atrophy. Motoneurons in the lumbar spinal cord can be impaired by direct injury, but are far more commonly indirectly impaired after an “above-level injury”, where the injury occurs above the lumbar level; such above-level injuries account for 90% of all SCIs in human patients (National Spinal Cord Injury Statistical Center (NSCISC), 2018). Lesions caused by these injuries damage descending motor and propriospinal tracts, resulting in dendritic atrophy in the lumbar motoneurons, muscle atrophy, and concomitant locomotor deficits (Byers et al., 2012; Liu et al., 2014a, b; Sengelaub et al., 2018). Surviving motoneu-

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doi: 10.4103/1673-5374.233434

Accepted: 2018-04-19

rons are thus a potential therapeutic target, and developing the ability to protect them from secondary atrophy is an important goal. As there are currently no effective treatments to preserve or restore lost function following SCI, identification of approaches that result in spared tissues/cell populations that may subsequently be the targets of regenerative therapy and/or rehabilitative plasticity interventions would be significant.

We reasoned that protecting spinal motoneurons from SCI-induced atrophy would have beneficial effects, and for the past several years we have been exploring novel treatment strategies to protect surviving motoneurons after SCI. In this review, we briefly summarize our work in a clinically relevant model in rats using steroid gonadal hormones as a powerful neurotherapeutic approach in the treatment of the secondary effects of spinal cord injury.

## Neuroprotection with Androgens and Estrogens

Androgens and estrogens have been demonstrated to powerful neuroprotective effects after a wide variety of neural injuries (Foecking et al., 2015; Brotfain et al., 2016). For example, both testosterone and estradiol protect against cell death (Pike, 2001; Yune et al., 2004), promote functional recovery (Jones et al., 2001; Sribnick et al., 2010), and stimulate motoneuron axonal growth after peripheral nerve injury (Kujawa et al., 1989; Islamov, et al., 2003). The mechanisms through which androgens and estrogens act are multiple, and include regulation of apoptosis (Fargo et al., 2009; Kachadroka et al., 2010), injury-induced upregulation of glial fibrillary acidic protein (GFAP; Jones et al., 1997; Samantaray et al., 2016), and mediation of the glial response (Jones et al., 1999; Ritz and Hausmann, 2008). Proteins thought to be involved in neuroprotection are also regulated by androgens and estrogens, including proteins with antioxidant or pro-inflammatory functions (Ahlbom et al., 2001; Nilsen, 2008; Ritz and Hausmann, 2008) and the neurotrophin brain-derived neurotrophic factor (BDNF; Solum and Handa, 2002; Verhovshek et al., 2010).

Gonadal steroid hormones provide protection from many of the pathophysiological changes specifically seen after SCI, for example reducing the inflammation and free radical generation that contribute to progressive secondary injury. After SCI, treatment of rats with estradiol resulted in improved motor function, reduced inflammation, attenuated apoptotic cell death, reduced lesion size, increased white matter sparing, and earlier cytokine release and astroglial response (Yune et al., 2004; Sribnick et al., 2005, 2010; Ritz and Hausmann, 2008; Kachadroka et al., 2010; Brotfain et al., 2016; Samantaray et al., 2016). Similarly, treatment with testosterone improves motor function in spinal cord injury patients. Patients treated with testosterone had higher American Spinal Injury Association (ASIA) discharge motor scores, a result ascribed to either improved strength through the anabolic effects of testosterone on skeletal muscle or its neuroprotective effects (Clark et al., 2008).

## Spinal Lesions

Consistent with previous studies, in our work we demonstrated that following contusion, the focal injuries delivered to the spinal cord developed into large lesions that spanned multiple thoracic spinal segments. Also consistent with previous studies (Yune et al., 2004; Sribnick et al., 2005; Chaovich et al., 2006; Ritz and Hausmann, 2008; Kachadroka et al., 2010; Siriphorn et al., 2012; Mosquera et al., 2014; Samantaray et al., 2016), treatment with estradiol was effective in reducing lesion volume; lesion volumes in animals treated only with estradiol were significantly smaller than those of all other groups (Sengelaub et al., 2018). This reduction in lesion size is thought to be the result of reducing inflammation, reactive astrogliosis, decreased immune response, apoptotic cell death, or reductions in oxidative stress (Yune et al., 2004; Ritz and Hausmann, 2008; Kachadroka et al., 2010; Siriphorn et al., 2012; Mosquera et al., 2014; Samantaray et al., 2016). Importantly, the reduction in lesion size we observed was produced through a physiological dose of estradiol, a result similar that reported by Samantaray et al. (2016) with low doses of estradiol. The efficacy of low dosages indicates that estradiol could be a promising therapeutic agent for treating SCI (Samantaray et al., 2016). Furthermore, in our work, estradiol was administered after trauma, modeling a clinically relevant situation.

In contrast, treatment with androgens, either alone or when combined with estradiol, proved to be ineffective in reducing lesion size. Four weeks of treatment with testosterone, dihydrotestosterone, or dihydrotestosterone combined with estradiol had no effect on reducing lesion volume or increased tissue sparing (Byers et al., 2012; Sengelaub et al., 2018). Curiously, the effect of estradiol on decreasing lesion volume was not present when estradiol was co-administered with dihydrotestosterone. This negation of the protective effect of estradiol is similar to that reported by Hauben et al. (2002), wherein treatment of female rats with dihydrotestosterone prior to SCI impaired recovery. Given that androgens have been demonstrated to regulate many of the same neuroprotective effects seen with estradiol treatment, *e.g.*, protecting against cell death (Pike, 2001), upregulating GFAP (Jones et al., 1997; Coers et al., 2002) or mediating the central glial response after injury (Jones et al., 1999), this negation with combined treatment after SCI warrants further study. One plausible mechanism for this negation with combined treatment could be through an androgen-mediated immunosuppression (Grossman, 1984). Regardless, given that testosterone is routinely metabolized into both estrogenic and androgenic metabolites, this negation could underlie the failure of testosterone treatment to affect SCI lesion volume we previously reported (Sengelaub et al., 2018).

## Neuromuscular Protection after SCI

Although extensive, the spinal lesions produced in our studies did not extend into the lumbar spinal cord, thus sparing the gray matter and resident motoneurons. We selected lumbar motoneurons innervating the quadriceps muscle as

our population of interest because of the major weight-bearing role this muscle plays. Counts of either Nissl-stained or retrogradely-labeled quadriceps motoneurons in SCI animals did not differ from those of sham animals, confirming that the lumbar motoneurons were not directly damaged by SCI-induced lesions. Similarly, soma size of quadriceps motoneurons was not significantly affected by SCI. Although quadriceps motoneuron number or soma size were unaffected after SCI, dendritic length in these motoneurons underwent marked dendritic atrophy (**Figure 1**). Dendritic length decreased by over 50% SCI animals compared to that of sham animals (**Figure 2A**). Reductions in dendritic length occurred throughout the radial distribution in SCI animals compared to sham animals, and were especially pronounced ventromedially where quadriceps motoneuron dendrites normally have a dense ramification into lamina VIII (**Figure 2B**). It is likely that the dendritic atrophy we observed following SCI in untreated animals reflects deafferentation resulting from the loss of descending motor and propriospinal tracts. Because both reticulospinal and propriospinal projections are concentrated in this area (Motorina, 1977; Jones and Yang, 1985; Menétey et al., 1985), the extensive lesions present after SCI could have produced a major denervation of dendrites in this area, resulting in the pronounced dendritic atrophy we observed. This loss is of particular significance after SCI, as descending reticulospinal fibers course through the ventral and lateral funiculi (Jones and Yang, 1985; Martin et al., 1985), and disruption of these tracts results in hindlimb motor deficits (Magnuson et al., 1999; Loy et al., 2002).

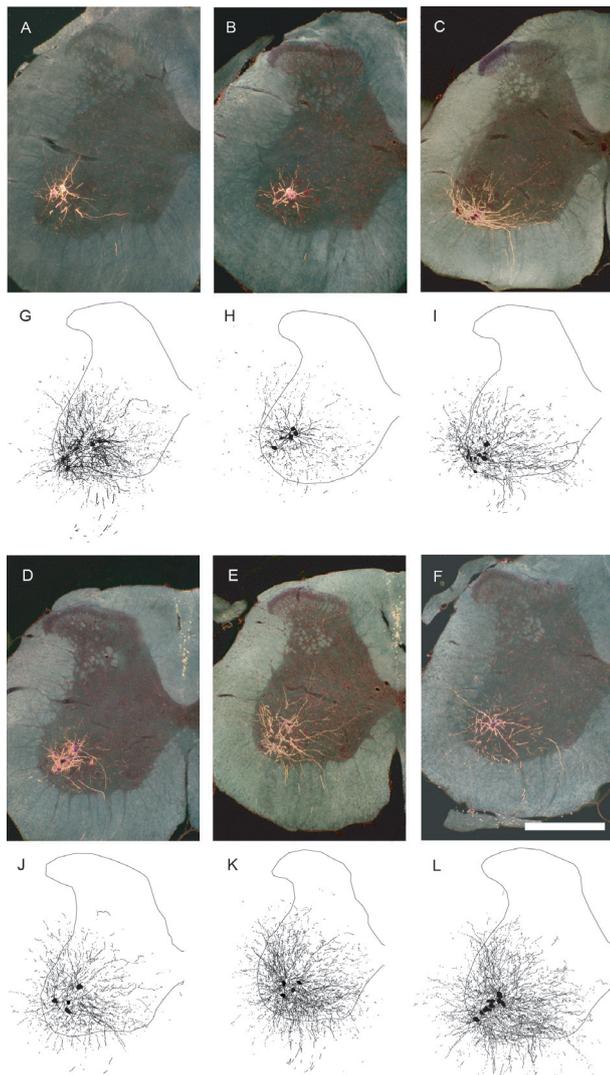
We further demonstrated that SCI-induced atrophy of quadriceps motoneuron dendrites was attenuated in estradiol-, dihydrotestosterone-, estradiol combined with dihydrotestosterone-, and testosterone-treated animals, and dendritic lengths in hormone-treated SCI groups did not differ from those of sham animals. Dendritic lengths in hormone-treated SCI groups were also significantly longer than those of untreated SCI animals by at least 57%. Interestingly, similar effects on dendritic length were present after treatment with androgens alone or in combination with estradiol, despite there being no reductions in lesion size or increases in tissue sparing in these groups (see above).

Because these effects were seen independent of lesion size, our results suggest that these hormonal effects could potentially be the result of local action on spinal circuitry below the level of the lesion. It is likely that the attenuation in SCI-induced dendritic atrophy we observed could have been produced by a hormone-mediated sprouting of motoneuron dendrites locally onto remaining afferents. Sprouting could potentially maintain motor activation, and such an effect of hormones on attenuating dendritic atrophy and supporting motoneuron activation has in fact been directly demonstrated (Fargo et al., 2009; Little et al., 2009; Foecking et al., 2015). The mechanisms responsible for this sprouting are not clear, but gonadal hormones have been shown to regulate the expression of cytoskeletal proteins (e.g.,  $\beta$ -tubu-

lin, Jones and Oblinger, 1994; Matsumoto et al., 1994; Jones et al., 1999; Brown et al., 2001; actin and microtubule-associated protein 2, Hansberg-Pastor et al., 2015), as well as neuritin, a critical downstream mediator of the ability of gonadal hormones to increase neurite outgrowth (Marron et al., 2005; Fargo et al., 2008a, b). Sprouting could be driven by direct action on the motoneurons or *via* indirect action on afferents. Thus, it is possible that a hormone-mediated protection of local spinal circuitry below the level of the lesion could be responsible for the motoneurons dendritic protection we observed. One possible protected spinal population could be the short axon propriospinal neurons, which provide the largest source of input to lumbar spinal motoneurons (Szentagothai, 1951; Sterling and Kuypers, 1968; Rustioni et al., 1971). Changes in these afferents could underlie the regressive changes we have observed in motoneurons after SCI. Afferent input to motoneurons is important for the maintenance of their dendritic morphology, and deafferentation of motoneurons results in dendritic retraction (Bernstein and Standler, 1983; Bernstein et al., 1984; Standler and Bernstein, 1984); the rescue of the major afferent source to motoneurons could underlie the beneficial effects of hormone treatment on motoneuron dendrites we have observed.

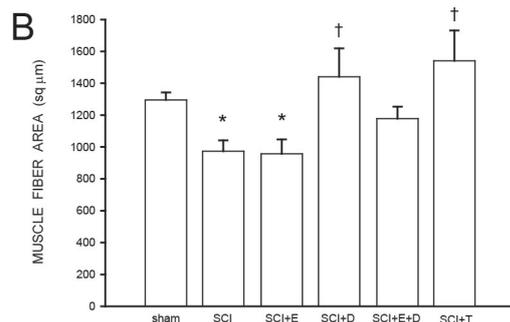
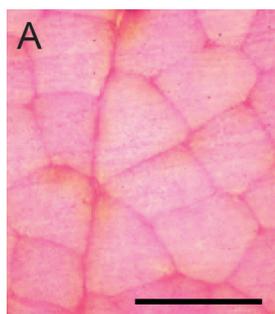
Following SCI, we found that quadriceps muscle fiber cross-sectional area in untreated SCI animals was decreased by 25%, typical of muscles innervated by motoneurons below the level of the lesion, especially in weight-bearing muscles such as the quadriceps (Peckham et al., 1976; Giangregorio and McCartney, 2006; **Figure 3**). Muscle atrophy after SCI can result from either muscle denervation due to a loss of motoneurons or disuse consequent to decreases in muscle activation potentially due to the loss of synaptic input to remaining motoneurons (Gordan and Mao, 1994). The atrophy we observed in our work cannot be ascribed to an effect of denervation, as we observed no changes in quadriceps motoneuron number, or the number of horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP)-labeled quadriceps motoneurons between sham animals and untreated SCI animals. Thus, the decreased fiber size we observed most likely reflects a disuse atrophy, potentially resulting after damage to descending and propriospinal projections and/or the reductions in quadriceps motoneuron dendritic length we observed. Such reductions in quadriceps motoneuron dendritic length result in attenuation of motor activation, reducing response amplitudes in the femoral nerve generated by dorsal root afferent stimulation (Little et al., 2009). Alternatively, disuse atrophy may also result from changes in muscle length or loading conditions that could decrease protein synthesis and increase protein degradation (Williams and Goldspink, 1973; Goldspink, 1978).

We found that estradiol treatment was ineffective in preventing muscle fiber atrophy, with areas decreasing 26% after SCI. Although estrogens have a variety of effects in skeletal muscle (e.g., downregulation of proinflammatory cytokines, enhancing insulin-like growth factor-1 (IGF-1)



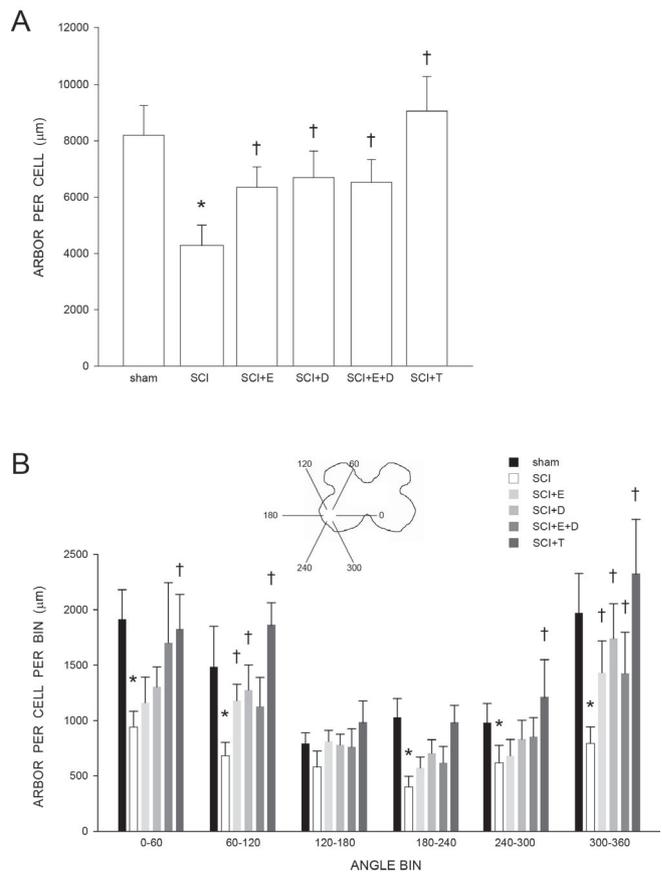
**Figure 1 Motoneuron morphology is protected by gonadal hormones following spinal cord injury.**

Darkfield digital micrographs and matching computer-generated composites of transverse hemisections through the lumbar spinal cords of a sham animal (A, G), an injured animal given a blank implant (SCI; B, H), an estradiol-treated injured animal (SCI + E; C, I), a dihydrotestosterone-treated injured animal (SCI + D; D, J), an injured animal treated with both hormones (SCI + E + D; E, K), and a testosterone-treated injured animal (SCI + T; F, L), after horseradish peroxidase conjugated to the cholera toxin B subunit (BHRP) injection into the left vastus lateralis muscle. Computer-generated composites of BHRP-labeled somata and processes were drawn at 480  $\mu\text{m}$  intervals through the entire rostrocaudal extent of the quadriceps motor pool; these composites were selected because they are representative of their respective group average dendritic lengths. Scale bar: 500  $\mu\text{m}$ . (Images from Byers et al. (2012) and Sengelaub et al. (2018)).



**Figure 3 Muscle fiber area is protected by androgens following spinal cord injury.**

(A) Cross-section through quadriceps muscle fibers. Scale bar: 100  $\mu\text{m}$ . (B) SCI reduces muscle fiber area; treatment with estradiol (SCI + E) is ineffective, but this reduction is prevented by treatment with dihydrotestosterone (SCI + D), alone or in combination with estradiol (SCI + E + D), or testosterone (SCI + T). Bar heights represent means  $\pm$  SEM. \*significantly different from sham, † significantly different from untreated SCI. (Data from Byers et al. (2012) and Sengelaub et al. (2018)).



**Figure 2 Motoneuron dendritic length and distribution is protected by gonadal hormones following spinal cord injury.**

(A) Dendritic lengths of quadriceps motoneurons of sham animals and injured animals that were either untreated (SCI), or treated with estradiol (SCI + E), dihydrotestosterone (SCI + DHT), estradiol and dihydrotestosterone combined (SCI + E + DHT), or testosterone (SCI + T). Following contusion injury, surviving quadriceps motoneurons lost over 50% of their dendritic length. Treatment with hormones attenuated this dendritic atrophy. (B) Inset: Drawing of spinal gray matter divided into radial sectors for measure of quadriceps motoneuron dendritic distribution. Quadriceps motoneuron dendritic arbors normally display a non-uniform distribution, with the majority of the arbor located between 300° and 120°. Following contusion injury, surviving quadriceps motoneurons in untreated animals (SCI) had reduced dendritic lengths throughout the radial distribution, especially ventromedially (60°, 300° to 360°). Treatment with hormones attenuated these reductions. Bar heights represent the mean  $\pm$  SEM. \*indicates significantly different from sham animals, † indicates significantly different from untreated SCI. (Data from Byers et al. (2012) and Sengelaub et al. (2018)).

expression, or satellite cell activation and proliferation; Tiidus et al., 2013), their effects on muscle fiber cross-sectional area vary in different muscles and in different directions. Estradiol replacement after ovariectomy has been reported to increase muscle fiber size in the gastrocnemius (Sciote et al., 2001), decrease it in the extensor digitorum longus (Suzuki and Yamamuro, 1985) and plantaris (Piccone et al., 2005), or either increase (Weigt et al., 2015) or decrease (Suzuki and Yamamuro, 1985) fiber size in the soleus.

In contrast, we also found that treatment with testosterone or dihydrotestosterone (either alone or in combination with estradiol) attenuated SCI-induced muscle fiber atrophy. These effects are consistent with the known protein anabolic effects of androgens on skeletal muscle tissue (Kochakian, 1975; Gao, 2010). Thus, treatment with androgens might have supported muscle protein synthesis and decreased protein degradation, and the resultant decrease in protein turnover could have prevented muscle atrophy. Alternatively, androgen treatment could have potentially altered mobility or activity in the treated animals, resulting in the preservation of both muscle as well as the related spinal cord circuitry and motoneuron dendritic morphology. This is quite plausible, as limb exercise after spinal cord transection during postnatal development has in fact been shown to prevent dendritic atrophy in spinal motoneurons (Gazula et al., 2004). Furthermore, exercise is known to elevate the expression of neurotrophic factors (e.g., BDNF) that can promote dendritic and axonal regrowth (Byers et al., 2012; Wilhelm et al., 2012; Sengelaub et al., 2018).

## Summary

Overall, our results provided the first evidence of pronounced dendritic atrophy in spinal motoneurons caudal to a contusive injury. More importantly, such atrophy was prevented with treatment with gonadal hormones, supporting their protective role after SCI. Together, our results indicate that the use of gonadal hormones could be an effective treatment after SCI, directed by the particular therapeutic goals. We believe that our work will lead to developing sex-appropriate hormone treatments that will be effective in treating multiple sequelae of SCI.

**Author contributions:** *The authors contributed equally to this study. DRS is the guarantor.*

**Conflicts of interest:** *None declared.*

**Financial support:** *This work was supported by grants from Indiana Spinal Cord and Brain Injury Research Fund (ISCBIRF) and by IU's Office of the Vice Provost for Research through the Faculty Research Support Program to DRS, and NIH R01 NS103481, R01 NS100531, Department of Veterans Affairs I01 RX002356, I01 BX003705, Craig H Neilsen Foundation 296749, Indiana Department of Health 019919, ISCBIRF, and Mari Hulman George Endowment Fund to XMX.*

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**Open peer reviewers:** *Meng-Jen Lee, Chaoyang University of Technology, China; Paul Lu, University of California San Diego, USA.*

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